Journal of Surgical Oncology 1998;69:168–172

Expression of Cyclooxygenase-2 Protein in Gastric Adenocarcinoma

KAZUHIKO UEFUJI, MD,[1]* TAKASHI ICHIKURA, MD,[1] HIDETAKA MOCHIZUKI, MD,[1] AND NARIYOSHI SHINOMIYA, MD[2]

[1]*Department of Surgery I, National Defense Medical College, Tokorozawa, Japan*
[2]*Department of Microbiology, National Defense Medical College, Tokorozawa, Japan*

Background and Objectives: Epidemiological studies have suggested that the regular use of nonsteroidal antiinflammatory drugs, which inhibit cyclooxygenase (COX), reduces the risk of colon cancer. The inducible COX-2 isoform has been reported to be upregulated in colorectal carcinomas and may play a role in colorectal carcinogenesis. The purpose of this study was to investigate the expression of COX-2 protein in human gastric adenocarcinomas.
Methods: COX-2 protein expression was examined in 23 patients with gastric adenocarcinoma by immunoblotting and immunohistochemistry.
Results: There was an increase in COX-2 protein levels in 19 of the 23 carcinomas (83%) compared with the paired normal gastric mucosa by an immunoblot analysis. There was no correlation between tumor histology and COX-2 protein expression. An immunohistochemical study in the 19 cases showed diffuse COX-2 staining in the cytoplasm of cancer cells. Mononuclear cells or fibroblasts of the cancer stroma were not stained with COX-2. Sporadic staining for COX-2 was observed in the normal fundic or metaplastic glandular cells in all cases.
Conclusions: COX-2 protein expression was elevated in most human gastric adenocarcinomas in comparison to the normal mucosa. COX-2 may therefore play an important role in gastric carcinogenesis.
J. Surg. Oncol. 1998;69:168–172.

KEY WORDS: cyclooxygenase-2; gastric adenocarcinoma; nonsteroidal anti-inflammatory drugs

INTRODUCTION

Cyclooxygenase (COX) is a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (PGs) [1]. Two isoforms of COX, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in most tissues and catalyzes the production of PGs for such normal physiological functions as cytoprotection in the stomach and vasodilation in the kidney [2–4]. On the other hand, COX-2 is not constitutively expressed but is induced by a variety of extracellular stimuli, such as tetradecanoylphorbol acetate, epidermal growth factor, and interleukin-1β [5–8]. COX-2 expression is thus elevated at inflammation sites [9].

Chronic intake of nonsteroidal antiinflammatory drugs (NSAIDs), which inhibit COX activity, has been reported to reduce the incidence of human colon cancer by 40% [10–12]. NSAIDs have also been reported to reduce the size and number of adenomas in patients with familial adenomatous polyposis [13–15]. In rat models of colon carcinogenesis, NSAIDs have exhibited chemopreventive effects as judged by reductions in the number of tumor-bearing rats and tumors per rat [16–18]. Recently, several studies have shown elevated levels of COX-2 mRNA and polypeptide in human colon carcinoma compared with normal mucosa, thus suggesting that COX-2 may play a role in colon carcinogenesis [19–22]. However, it is unclear as to whether or not COX-2 protein is involved in the carcinogenesis of gastric carcinoma. The purpose of this study was to investigate the expression of

*Correspondence to: Kazuhiko Uefuji, Department of Surgery I, National Defense Medical College, 3-2 Namiki, Tokorozawa 359, Japan. Fax No.: (81) 42-996-5205.
Accepted 18 September 1998

COX-2 protein in human gastric adenocarcinomas by immunoblotting and immunohistochemical analyses.

MATERIALS AND METHODS

Patient Samples

Twenty-three patients undergoing surgery for primary gastric adenocarcinoma at the National Defense Medical College Hospital (Tokorozawa, Japan) from 1993 to 1998 were randomly selected. Paired samples of cancer tissue and normal gastric mucosa more than 5 cm away from the tumor were obtained from each patient at the time of surgery. Samples were frozen in liquid nitrogen immediately and stored at −80°C until used for immunoblot analysis. The remaining tissues were fixed in 10% formalin and embedded in paraffin for an immunohistochemical study and routine histological examination.

Immunoblot Analysis

Frozen tissue specimens were thawed, homogenized, and treated with 100% trichloroacetic acid on ice. Cellular fractions collected by centrifugation were treated with 9M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 10% lithium dodecylsulfate, and 2M Tris-HCl. After sonication and centrifugation, the supernatants were collected for protein samples and the protein concentration was measured. Protein samples (20 μg/lane) were separated on 10% SDS polyacrylamide gels, then electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was immersed in 0.5% skim milk overnight at 4°C for blocking. It was next incubated with a rabbit polyclonal immunoglobulin G (IgG) specific for human COX-2 (Immunobiological Laboratories, Fujioka, Japan) for 1 hr at room temperature and then with peroxidase-labeled goat antirabbit IgG for 1 hr at room temperature. Reaction bands were visualized by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The membrane image was incorporated into a computer and quantitated by using image software (National Institutes of Health, Bethesda, MD). The ratio of the densitometrically determined expression of COX-2 in cancer to paired normal gastric mucosa was evaluated in each patient.

Immunohistochemistry

Tissue samples embedded in paraffin were sectioned at a thickness of 4 μm, deparafinized, and rehydrated. Specimens were heated in an autoclave for 9 min at 90°C for antigen retrieval, immersed in 0.3% hydrogen peroxide in methanol for 30 min, and then immersed in normal goat serum for 30 min. Slides were incubated with a rabbit polyclonal IgG specific for human COX-2 (Immunobiological Laboratories) overnight at 4°C followed by the standard streptavidin-biotin immunoperoxidase method [23].

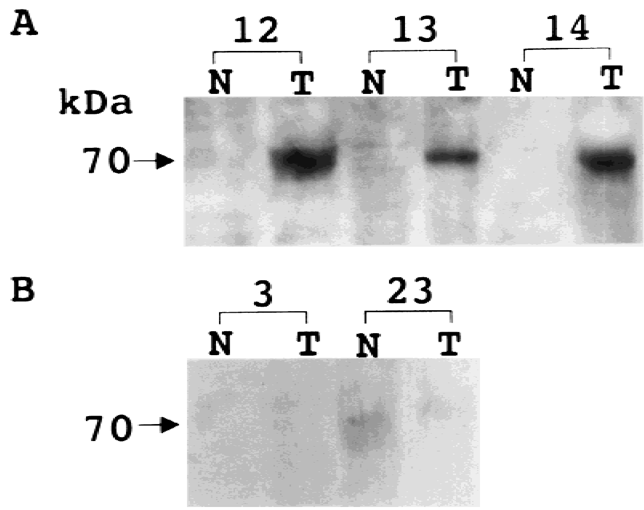

Fig. 1. A representative immunoblot analysis of cyclooxygenase-2 (COX-2) protein expression in normal gastric mucosa (N) and tumor (T) tissue. **A:** Samples from cases 12–14, with ratios of the densitometrically determined expression of COX-2 in cancer tissue to that in the paired normal gastric mucosa of 9.9, 5.9, and 15.9, respectively. **B:** Samples from cases 3 and 23, with a ratio of less than 1.0.

RESULTS

Cancer tissue showed intense immunoreactive bands of COX-2 protein, located at 70 kDa, in 19 of the 23 carcinomas (83%), although normal gastric mucosa showed low levels of COX-2 protein expression (Fig. 1). The ratio of the densitometrically determined expression of COX-2 in cancer to the paired normal mucosa was more than 1.0 in these 19 cases, ranging from 1.4 to 20 (Table I). The ratio of COX-2 in cancer to the paired normal mucosa was more than 1.0 in eight of 10 patients (80%) with intestinal-type carcinoma and in 11 of 13 patients (85%) with diffuse-type carcinoma according to Lauren's classification [24] (Table I).

Immunohistochemical detection of COX-2 protein showed diffuse staining in the cytoplasmic and perinuclear regions of cancer cells in the 19 patients with a ratio of COX-2 in cancer to paired normal mucosa of higher than 1.0 based on an immunoblot analysis (Fig. 2). Immunoreactive COX-2 in cancer cells was undetectable in the remaining four cases. Expression of COX-2 was observed neither in the inflammatory mononuclear cells nor in the fibroblasts of the cancer stroma. In the normal gastric mucosa, COX-2 was observed sporadically in the fundic or metaplastic glandular cells but not in the mucosal mononuclear cells (Fig. 3).

DISCUSSION

So far, two COX isoforms have been characterized, namely, COX-1 and COX-2. COX-1 has been reported to be expressed in the normal intestine, and its levels do not change in intestinal tumors [20]. In contrast, COX-2,

TABLE 1. Results of an Immunoblot Analysis for Cyclooxygenase-2 (COX-2) Expression

Case	Age (yr)	Sex	Location[a]	Depth[b]	Histologic type[c]	Ratio of COX-2 (Ca/Nor)[d]
1	44	M	M	ss	Diffuse	3.4
2	59	M	W	si	Diffuse	1.4
3	57	M	W	se	Diffuse	0.4
4	86	F	M	ss	Intestinal	0.9
5	68	M	C	se	Diffuse	0.7
6	55	M	M	mp	Intestinal	1.9
7	71	F	A	mp	Diffuse	1.7
8	58	F	A	ss	Intestinal	1.8
9	64	F	W	se	Diffuse	20
10	60	M	C	ss	Diffuse	3.8
11	48	M	M	mp	Intestinal	2.6
12	57	M	M	ss	Intestinal	9.9
13	51	F	M	se	Diffuse	5.9
14	78	M	A	se	Intestinal	15.9
15	73	M	W	se	Intestinal	9.1
16	83	F	A	se	Intestinal	2.6
17	80	F	A	se	Diffuse	2.4
18	39	F	M	se	Diffuse	3.6
19	54	M	C	ss	Diffuse	8.7
20	77	M	W	se	Intestinal	3.2
21	52	M	M	sm	Diffuse	2.2
22	62	M	A	ss	Diffuse	1.9
23	63	M	A	mp	Intestinal	0.4

[a]C, upper third; M, middle third; A, lower third; W, whole stomach.
[b]Depth of tumor invasion: sm, submucosa; mp, muscularis propria; ss, subserosa; se, serosa; si, tumor invading adjacent structures.
[c]Classification by Lauren [24].
[d]The ratio of densitometrically determined COX-2 protein expression in the carcinoma tissue to that in the paired normal gastric mucosa.

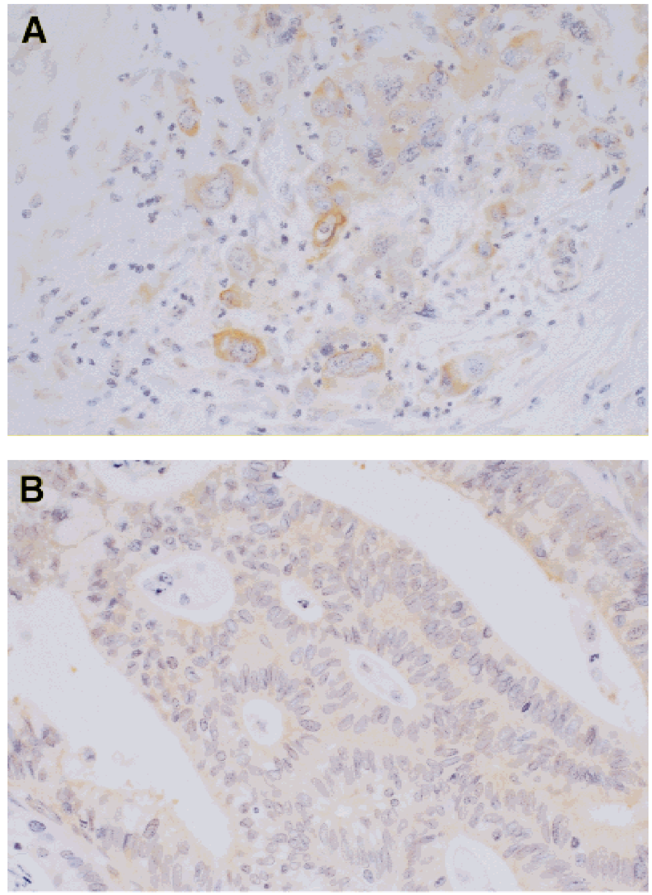

Fig. 2. Immunostaining for cyclooxygenase-2 (COX-2) in carcinoma tissue. Intense and diffuse staining for immunoreactive COX-2 was observed in both diffuse **(A)** and intestinal **(B)** types. No inflammatory mononuclear cells or fibroblasts of the cancer stroma were stained with COX-2.

which is undetectable in the normal intestine, has been reported to be upregulated in up to 85% of colorectal adenocarcinomas [19–22]. We hypothesized that COX-2 levels are elevated in gastric adenocarcinomas as well and, thus, examined COX-2 protein expression by immunoblot and immunohistochemical staining analyses.

In the immunoblot analysis, there was an upregulation of COX-2 protein expression in 19 of the 23 gastric carcinomas (83%) compared with the paired normal gastric mucosa. The degree of upregulation ranged from 1.4- to 20-fold, when the densitometrically determined expression of COX-2 in cancer tissue was compared with the paired normal mucosa. The main source of increased COX-2 protein in the cancer tissue seemed to be the cancer cells themselves because immunohistochemistry revealed COX-2 to be preferably expressed in the cancer cells but not in the mononuclear cells or fibroblasts of the cancer stroma. We found sporadic COX-2 immunostaining in the normal gastric mucosa, in accordance with the magnitude of the results of an immunoblot analysis. COX-2 mRNA levels have also been reported to be elevated in gastric cancer tissue [25]. However, the upregulation of COX-2 might not be a common feature of malignant transformation because no such event was detected in either human breast or ovarian carcinomas [20,25]. In our study, no obvious relationship was observed between COX-2 expression and cancer histology. The relationship between COX-2 expression and stage of the tumor was unresolved because of a limited number of patients with early gastric carcinomas.

COX-2 enzyme can contribute to carcinogenesis via several different mechanisms. PGE_2 formed by COX-2, which has been shown in the tissue of human colon or lung cancers [26–28], can suppress the local immunity against tumor cells [29]. Overexpression of COX-2 has been reported to inhibit apoptosis [30], which may prolong the survival of cells containing damaged DNA. The COX pathway of arachidonic acid metabolism is resp-ponsible for generating a direct-acting mutagen, malondialdehyde, which may induce mutations in the *p53* gene in the human colon [8,31]. Although we have not obtained sufficient data to determine the relationship between COX-2 expression and tumor stage or prognosis, COX-2 might enhance the metastatic activity of tumor cells. COX-2-derived thromboxane, a platelet proaggre-

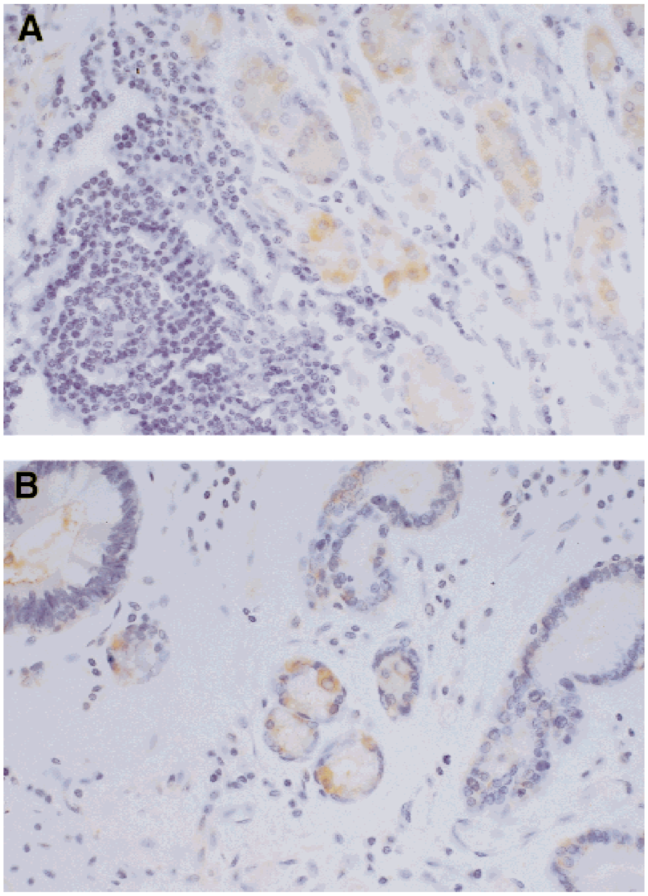

Fig. 3. Immunostaining for cyclooxygenase-2 (COX-2) in normal tissue. Sporadic expression of COX-2 is shown in fundic glandular cells **(A)** and metaplastic glandular cells **(B).** No mucosal mononuclear cells were stained with COX-2.

gatory factor [32], may facilitate tumor metastasis by platelet–tumor cell interactions [27]. COX-2-expressing colon cancer cells have been reported to possess a potent extracellular matrix-degrading activity due to the activation of matrix metalloproteinase-2, which may increase the metastatic potential [33]. The clinical significance of the expression of COX-2 in gastric cancer cells remains to be elucidated.

In conclusion, we have demonstrated elevated levels of COX-2 protein expression in most gastric adenocarcinomas compared with normal gastric mucosa. Further investigation is required, however, to determine the role of the COX-2 enzyme in the genesis and development of human gastric carcinoma.

REFERENCES

1. DeWitt DL: Prostaglandin endoperoxide synthase: Regulation of enzyme expression. Biochim Biophys Acta 1991;1083:121–134.
2. Kujubu DA, Reddy ST, Fletcher BS, et al.: Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. J Biol Chem 1993;268:5425–5430.
3. Kargman S, Charleson S, Cartwright M, et al.: Characterization of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. Gastroenterology 1996;111:445–454.
4. Harris RC, McKanna JA, Akai Y, et al.: Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. J Clin Invest 1994;94:2504–2510.
5. Kujubu DA, Fletcher BS, Varnium BC, et al.: TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 1991;266:12866–12874.
6. Hla T, Neilson K: Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA 1992;89:7384–7388.
7. Crofford LJ, Wilder RL, Ristimaki AP, et al.: Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues: Effect of interleukin-1β, phorbol ester, and corticosteroids. J Clin Invest 1994; 93:1095–1101.
8. Mestre JR, Subbaramaiah K, Sacks PG, et al.: Retinoids suppress epidermal growth factor-induced transcription of cyclooxygenase-2 in human oral squamous carcinoma cells. Cancer Res 1997; 57:2890–2895.
9. Masferrer JL, Zweifel BS, Manning PT, et al.: Selective inhibition of inducible cyclooxygenase-2 *in vivo* is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91:3228–3232.
10. Kune GA, Kune S, Watson LF: Colorectal cancer risk, chronic illnesses, operations, and medications: Case control results from Melbourne Colorectal Cancer Study. Cancer Res 1998;48:4399–4404.
11. Rosenberg L, Palmer JR, Zauber AG, et al.: A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of the large-bowel cancer. J Natl Cancer Inst 1991;83:355–358.
12. Marnett LJ: Aspirin and related nonsteroidal anti-inflammatory drugs as chemopreventive agents against colon cancer. Prev Med 1995;24:103–106.
13. Waddell WR, Loughry RW: Sulindac for polyposis of the colon. J Surg Oncol 1983;24:83–87.
14. Waddell WR, Gasner GF, Cerise EJ, et al.: Sulindac for polyposis of colon. Am J Surg 1989;157:175–178.
15. Labayle D, Fischer D, Vielh P, et al.: Sulindac causes regression of rectal polyps in familial adenomatous polyposis. Gastroenterology 1991;101:635–639.
16. Kudo T, Narisawa T, Abo S: Antitumor activity of indomethacin on methylazoxymethanol-induced large bowel tumors in rats. Gann 1980;71:260–264.
17. Narisawa T, Sato M, Tani M, et al.: Inhibition of development of methylnitrosourea-induced rat colon tumors by indomethacin. Cancer Res 1981;41:1954–1957.
18. Pollard M, Luckert PH: Prolonged antitumor effect of indomethacin on autochthonous intestinal tumor in rats. J Natl Cancer Inst 1983;70:1103–1105.
19. Eberhart CE, Coffey RJ, Radhika A, et al.: Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–1188.
20. Kargman SL, O'Neill GP, Vickers PJ, et al.: Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res 1995;55:2556–2559.
21. Sano H, Kawahito Y, Wilder RL, et al.: Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 1995;55: 3785–3789.
22. Kutchera W, Jones DA, Matsunami N, et al.: Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: Evidence for a transcriptional effect. Proc Natl Acad Sci USA 1996; 93:4816–4820.
23. Oka A, Takashima S: Induction of cyclo-oxygenase 2 in brains of patients with Down's syndrome and dementia of Alzheimer type: Specific localization in affected neurones and axons. Neuroreport 1997;8:1161–1164.
24. Lauren P: The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965; 64:31–49.
25. Ristimaki A, Honkanen N, Jankala H, et al.: Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 1997;57: 1276–1280.

26. Bennett A, Civier A, Hensby CN, et al.: Measurement of arachidonate and its metabolites extracted from human normal and malignant gastrointestinal tissues. Gut 1987;28:315–318.
27. Rigas B, Goldman IS, Levine L: Altered eicosanoid levels in human colon cancer. J Lab Clin Med 1993;122:518–523.
28. McLemore TL, Hubbard WC, Litterst CL, et al.: Proflies of prostaglandin biosynthesis in normal lung and tumor tissue from lung cancer patients. Cancer Res 1998;58:3140–3147.
29. Goodwin JS, Ceuppens J: Regulation of the immune response by prostaglandins. J Clin Immunol 1983;3:295–314.
30. Tsuji M, DuBois RN: Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.
31. Marnett LJ: Aspirin and potential roles of prostaglandins in colon cancer. Cancer Res 1992;52:5575–5589.
32. Hamberg M, Samuelsson B: Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proc Natl Acad Sci USA 1974;71:3400–3404.
33. Tsuji M, Kawano S, DuBois RN: Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 1997;94:3336–3340.